AD_____

Award Number: W81XWH-06-1-0551

TITLE: Characterizing the Dynamic Response of the Estrogen Receptor to Agonists and Antagonists by Multifrequency Electron Spin Resonance Spin-Labeling

PRINCIPAL INVESTIGATOR: David E. Budil

CONTRACTING ORGANIZATION: Northeastern University
Boston, MA 02115-5000

REPORT DATE: May 2008

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
31-05-2008	Annual	1 May 2007 - 30 Apr 2008

4. TITLE AND SUBTITLE

Characterizing the Dynamic Response of the Estrogen Receptor to Agonists and Antagonists by Multifrequency Electron Spin Resonance Spin-Labeling

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-06-1-0551

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
David E. Budil

Email: d.budil@neu.edu

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

Northeastern University
Boston MA 02115-5000

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

The overall objective of this project is to characterize the detailed structural and dynamic response of the estrogen receptor ligand binding domain (ER-LBD) to a variety of ligands ranging from strong estrogens to strong antiestrogens using electron spin labeling. The first two technical aims for this reporting period involved completing preparation of site-directed spin-labeled mutants of the ER-LBD and completing synthesis new spin-labeled ligands for the proposed studies. These tasks have essentially been completed and led to the development of a new fluorescence and EPR-based ER ligand binding assay. Towards the third technical aim of the reporting period, we have completed several initial EPR studies of ligand-dependent dynamics as a function of position in the ER as well as a series of spin-spin distance measurements. The results clearly support our initial hypothesis that the physical response of the ER protein to different ligand types can be resolved and characterized by EPR spin-labeling.

15. SUBJECT TERMS
None listed.

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
					USAMRMC
a. REPORT	b. ABSTRACT	c. THIS PAGE			19b. TELEPHONE NUMBER *(include area code)*
U	U	U	UU	14	

Table of Contents

2. Progress Report (Year 2)

Introduction

This proposal focuses on the key first steps in the estrogen response of breast cells, specifically the physical interaction between estrogen-like molecules and the ligand binding domain (LBD) of the ER. Although recent crystal structures of ER-LBD have implicated the C-terminal helix-12 (H12) of the ER in this response, X-ray analysis cannot characterize the dynamic behavior of H12 that is thought to play a major role in the tissue selectivity of the ER. By placing nitroxide spin labels at strategic points on the ER-LBD protein as well as on estrogenic ligands, we can map the dynamics and key distances in the complex over the entire range of ligand activities from estrogenic to antiestrogenic. This will afford the first characterization of the ER response under near physiological conditions, which will significantly aid the design of partially selective estrogen modulators for breast cancer therapies.

Specific aims for the second year involved completion of the synthesis of the necessary spin-labeled estrogen ligands, development and expression of the full range of single- and double- cysteine mutants of the ER-LBD for the proposed spin-labeling studies, and initiation of the ESR spectroscopic studies for both dynamics and distance measurements.

Task 1 *Synthesize a novel series of estrogenic probes with a nitroxide reporter group substituted at the 17α position and a short alkyl substituent at the 11β position to control the probe's activity (Months 1-24)*

Task 1a: 11β-substituted estrogen spin labels

We have completed all but the final "click" (Huisgen 1,3 dipolar cycloaddition) step needed to synthesize or the proposed 11β-substituted estrogenic ligands. The overall synthesis is summarized in **Figure 1**. The propargyl-substituted TEMPONE and corresponding estrogen-linked azide compounds are now in hand, and a small-scale click reaction has been carried out and shown to give a mixture of products including the correct

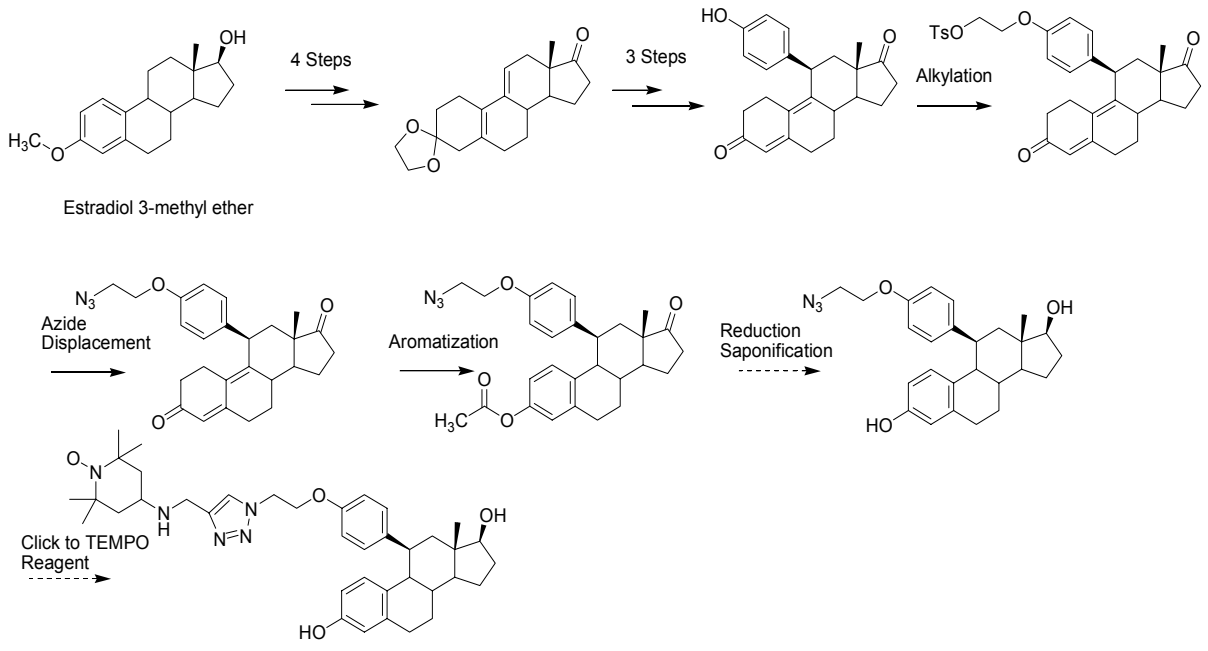

Figure 1: Outline of synthetic scheme towards 11β-substituted nitroxide-labeled estrogens

one. The immediate next steps will be to verify the characterization of product, optimize the yield, and scale up the reaction for spin-label studies.

Task 1b: Synthesis of 17α-substituted estrogen nitroxides

Our original proposal to synthesize 17α-substituted estrogen nitroxides was based on an extensive series of previous results in which large substituents at the 17α position were shown not to significantly reduce either the relative binding affinity (RBA) or the relative stimulatory activity (RSA) of the compound relative to estradiol. However, these studies were performed mainly with hydrocarbon and halide substituents. More recently we have found that a series of compounds with nitrogens in substituents at this position (*cf*. **Figure 2**) do exhibit attenuated RBA but dramatically lower biological activities than estradiol. the biological activity as shown in **Table 1** below. Thus, nitrogen appears to switch the compounds from being agonists to being inactive (not strictly antagonists). This unanticipated effect makes the pharmacology obtained with such substituents highly unpredictable, and significantly complicates the prediction of pharmaceutical effects from measurements made with 17α-labeled probes. In light of these findings, we have decided to concentrate on 11β-substituted probes for our distance measurement studies, which exhibit very strong relative binding affinities and commensurate biological activities.

Table 1. Relative binding affinity (RSA) and relative stimulatory activity of heterarylvinyl estradiols compared to estradiol. RBA estradiol = 100% and RSA estradiol = 100%

AR =	RBA+/-S.D.	RSA+/-S.D.
phenyl-**3a**	10.3 ± 2.9	9.5 ± 2.5
2-pyridyl-**3b**(SLO-1291)	1.5 ± 0.8	0.1 ± 0.07
3-pyridyl-**3c**(SLO-0562)	4.0 ± 1.0	0.25 ± 0.07
4-pyridyl-**3d**(SLO-1082)	2.5 ± 1.0	0.1 ± 0.1
5-pyrimidinyl-**3e**(SLO-0772)	0.8 ± 0.3	0.4 ± 0.2
2-thienyl-**3f**(SLO-0370)	2.7 ± 1.2	2.5 ± 0.1
3-thienyl-**3g**(SLO-0459)	2.9 ± 0.4	2.5 ± 0.8

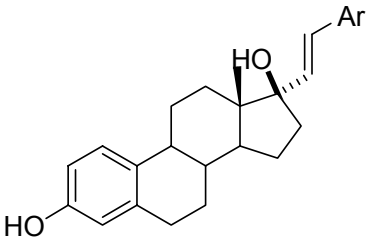

Figure 2 Base structure of 17α heterarylvinyl estradiols

Despite these disadvantages, we have demonstrated that 17α-substituted nitroxide compounds do have unexpected utility for binding assays, as we describe below.

We have extended our studies to include ERα bound to a series of estradiols labeled at the 17α position, the structures of which are shown in **Figure 2**.

Task 1c: Binding studies

Our preliminary studies have focused on the detailed interactions of the HO2453 probe with the ERα-LBD. **Figure 3** shows a binding study holding the spin label concentration constant at 50 μM while varying the protein concentration. At higher protein concentrations the narrow three-line spectrum of the unbound probe is replaced by a broad

signal of immobilized probe, demonstrating that the probe does bind to the receptor. A binding curve can be constructed simply by measuring the intensity of a line from the mobile species, as shown in **Figure 4.** The approximate dissociation constant K_d of HO2453 appears to be about 20 μM (compared to about 1-10 nM for estradiol).

We have discovered that the nitroxide quenches the fluorescence of two or three tryptophan residues that are in the estrogen binding pocket of the ERα protein. This is a good indication that the label is going into the actual ligand site of the protein, and also provides a very useful new and highly sensitive binding assay as an alternative to the standard radiolabel assay. **Figure 5** shows the fluorescence intensity of the sample as a function of estrogenic nitroxide ligand concentration, which gives a dissociation constant K_d that is quite close to that estimated from the ESR.

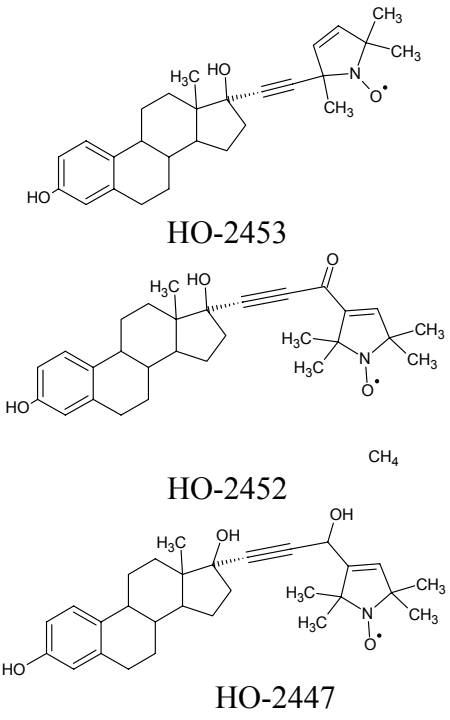

Figure 3. 17α nitroxide-labeled estradiols used in this study.

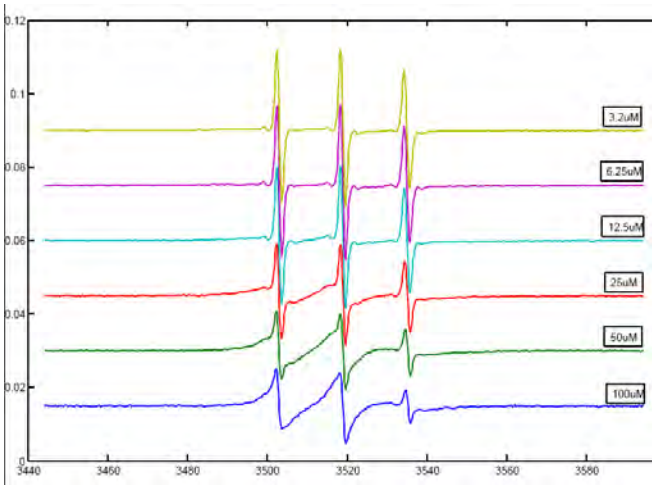

Figure 3: ESR binding study of HO-2453 probe to ERα-LBD. Probe concentration 50μM, protein concentration as indicated.

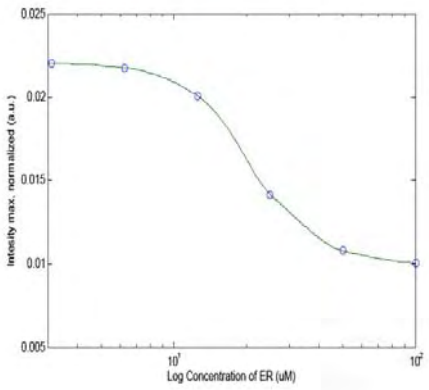

Figure 4: Intensity of low field hyperfine line as a function of ER concentration.

Additional complications were observed with the HO-2452 probe. Mass spectroscopic data indicates that this compound can form covalent attachments to the ERα, most likely via attachment of the alkynyl group to nucleophilic side chains.

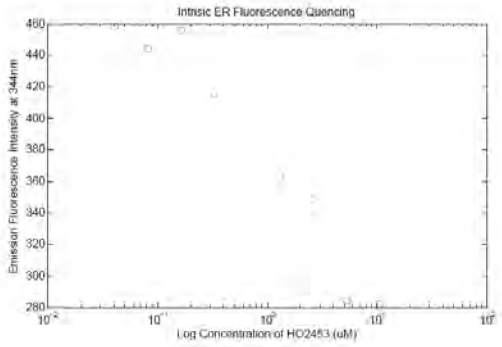

Figure 5: Fluorescence emission of ER at 344nm as a function of HO2453 concentration.

Task 2: *Generate a series of site-directed spin-labeled mutants of the estrogen receptor α isoform (ERα) with labeling sites near the putative flexible Helix 12 region consisting of residues 538-548 (Months 1-24).*

Table 2 below summarizes our second year progress towards developing the range of ER-LBD mutants outlined in our proposal. In addition to the WT, M_0, M_1, and M_8 mutants expressed in Year 1, we are now able to express and label M_2, M_5, and M_9 at the same high yields as the other mutations. Mass spectroscopy of some of the proteins produced in Year 1 revealed a second mutation away from the label site was accidentally introduced during mutagenesis that has now been corrected. In our initial characterization of the proteins be mass spectroscopy as well as in the initial spin label studies of the proteins, we observed several effects that were attributable to the presence of bound detergent molecules introduced during the extraction steps Our protocol for preparing protein has been modified to avoid these difficulties.

As an adjunct to our proposed work, we are also now providing ERα samples to the laboratory of our departmental colleague, Prof. John Engen, for corroborative studies of ER dynamics using hydrogen deuterium exchange mass spectrometry (HDXMS). The more detailed mass spectroscopic characterization provided by his lab also affords an additional level of quality control for our sample preparation.

Our initial work plan identified a range of possible label sites along the H_{12} region of the protein, depending upon whether any interference or other difficulties were observed at the 543 label site selected for initial studies. Since the initial studies demonstrate that this site is suitable for the planned distance measurements in Year 3, we have not pursued expression of the other label sites in the 539-543 range. This Task is therefore essentially completed.

Table 2: Mutants of the ERα-LBD used in the present study

	Mutation	Description	Usage	Label site(s)	Status
WT	none	wild type	standard/control binding assays,	381+417+ 350	expressed
M_0	C381S/C417S/ C530S	Cys-free mutant	"blank" Cys-free mutant	none	expressed
M_1	C381S/C417S	hinge label	hinge dynamics	530	expressed labeled
M_2	C381S/C530S	protein body label	control for dynamics measurements	417	expressed, labeled
M_3	M_1 + L539C	hinge+H_{12}	H_{12} distance measurement	530+539	not constructed
M_4	M_1 + L541C	hinge+H_{12}	H_{12} distance measurement	530+541	not constructed
M_5	M_1 + M543C	hinge+H_{12}	H_{12} distance measurement	530+543	expressed, labeled
M_6	M_0 + L539C	label H_{12}	H_{12} dynamics	539	not constructed
M_7	M_0 + L541C	label H_{12}	H_{12} dynamics	541	not constructed
M_8	M_0 + L543C	label H_{12}	H_{12} dynamics	543	expressed, labeled
M_9	C381S	hinge+body	H_{12} distance measurement	417+530	expressed, labeled

Task 3: Characterize by EPR spectroscopy the specific structural differences that accompany binding of agonists and antagonists in complexes of spin-labeled estrogens to ER that is also spin-labeled.

Task 3a: cw-EPR spectroscopy of singly labeled ER mobility

We have completed a cw-EPR study of the mobility at one location of the ER-LBD (label site 530) for a series of ligands ranging from estradiol to full antagonists. **Figure 6** illustrates the qualitative changes that occur in the cw-EPR spectrum that reflect local probe ordering. The splitting and sharpness of the outer peaks in the ESR spectrum (red lines) reflect the degree of ordering at the label location. The native agonist estradiol exhibits relatively high probe ordering, whereas differences appear in these peaks in the absence of ligand (Apo) or an antagonist bound. The specific ligands used in this study are summarized in **Figure 7.**

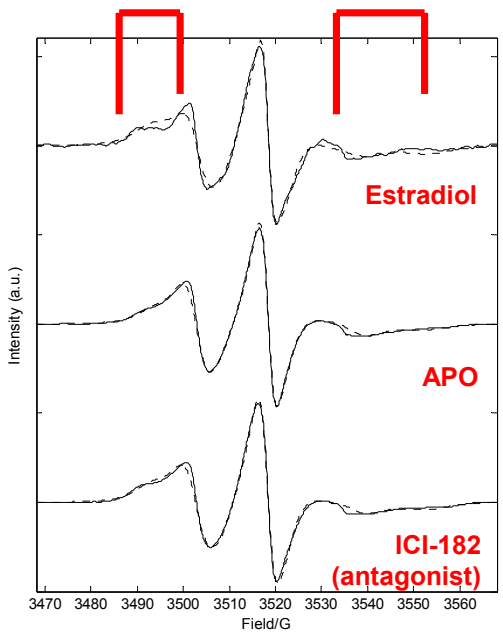

Figure 6: X-band cw-ESR spectra of ERα labeled at residue 530 bound with estradiol, an agonist and an antagonist.

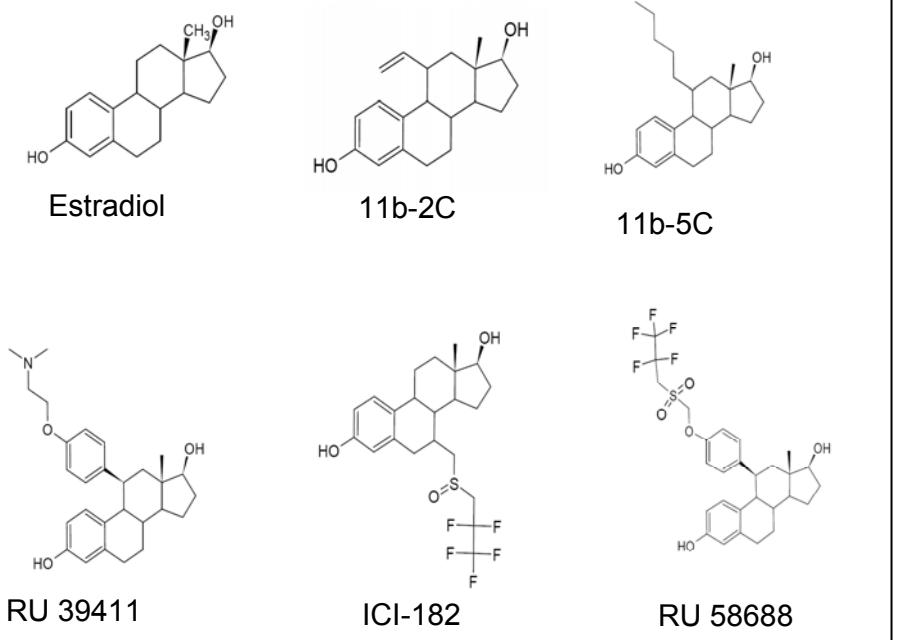

Figure 7 ER ligands used in single-label mobility study

A more quantitative measure of local dynamics is obtained by careful least-squares fitting of the ESR lineshape (fits shown by dotted lines, Figure 6). The important fitting parameters, summarized in **Table 3**, include the rotational diffusion rate and orienting potential coefficients $c_{L,K}$, where the local orienting potential of the spin probe is expressed as an expansion of real-valued spherical harmonics,

$$U(\theta,\phi) = \sum_{L,K\,2,4} c_{L,K} Y_K^L(\theta,\phi)$$

The results show that the dynamic motion of the probe is relative unaffected by the ligand, whereas the degree of ordering changes significantly

Table 3 Results from least-squres fitting of cw-EPR spectra of ER-LBD labeled at Site 530 for different

ligand	**$\log_{10} R_\perp$**	**$c_{2,0}/kT$**	**$c_{2,2}/kT$**	**$c_{4,0}/kT$**	**$c_{4,2}/kT$**
APO	7.52	1.1	0.66	0.31	0.39
E_2	7.56	1.44	1.07	0.89	0.38
11-2C	7.55	1.35	1.1	0.81	0.32
11-5C	7.55	1.38	1	0.86	0.3
RU39411	7.54	1.36	1.16	0.41	0.25
RU58688	7.49	1.15	1.17	0.61	0.46
ICI-182	7.51	1.48	1.03	0.61	0.47

The ordering results given in Table 3 are more easily visualized in **Figure 8,** which shows a series of intensity plots that depict the orientational distribution of an axis that is fixed in the protein frame relative to the molecular frame of the spin label for each of the ligands studies. The width of the distribution gives a clear visual indication of the

progression from high ordering (small spot) to greater disorder as one proceeds from the native agonist estradiol to a strong antagonist, ICI-182. The results clearly demonstrate our ability to characterize subtle changes in the ER-LBD in response to different ligands.

The initial study presented here focused on site 530 because it was the first available single cysteine mutant to be studied. However, from its location at the hinge region of H12, one might expect relatively subtle changes at this label site in comparison to labels on the H12 domain. Our ability to resolve such subtle changes by EPR gives us confidence that we will be able to observe dramatic effects at the other label locations to be studied in Year 3.

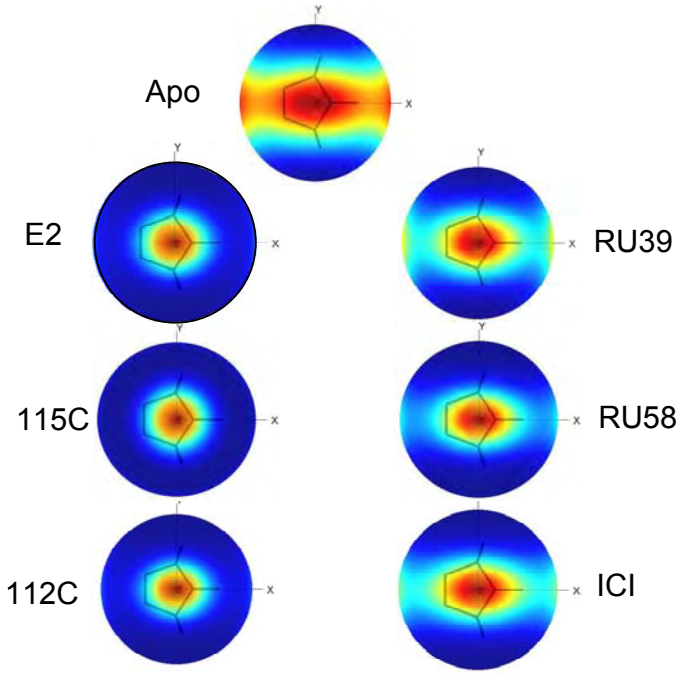

Figure 8 Spherical intensity plots of the orientation distribution of spin label at residue 530 of ERα in the presence of binding of different agonists (left) and antagonists (right)

Task 3d: Distance measurements in doubly-labeled systems

Continuous-wave EPR distance measurements

We have used the HO-2105 estradiol bound to ER spin labeled at position 530 to measure interspin distances between the bound estradiol probe and the hinge region of the H12. We were able to measure this distance using local instrumentation and the dipolar deconvolution method. Briefly, the dipolar-broadened spectrum D(B) (green line, **Figure 9)** is treated as the convolution between the spectrum of a single label S(B) (red line, Figure 9) and a broadening function M(B): D(B)=S(B)*M(B). The width of the broadening function $\langle 2B \rangle$, defined as

$$\langle 2B \rangle = \frac{\int_{-\infty}^{\infty} |2B| M(B) dB}{\int_{-\infty}^{\infty} M(B) dB}$$

gives an estimate of the average spin-spin distance $\langle r \rangle$ according to the equation

$$\langle r \rangle = \left(\frac{9}{8} \frac{g_e \beta}{\langle 2B \rangle} \right)^{1/3}$$

Using the dipolar deconvolution method we measured a distance of 16±1Å, which agrees quite well with the 17 Å measured from a molecular model derived from the ligand binding domain crystal structure. This result indicates that the overall \ structure of the ligand binding domain in solution is quite similar to that deduced by X-ray crystallography.

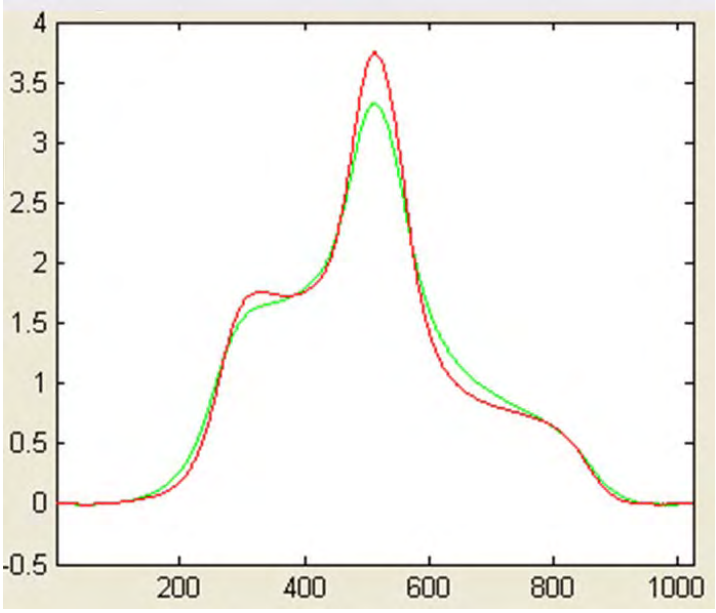

Figure 9 Normalized EPR rigid limit spectra of doubly labeled (green) and noninteracting singly labeled (red) ERα

Double electron-electron resonance (DEER) *distance measurements*

We have initiated a series of experiments in which two spin labels are bound at different locations in the ER and the distance between them measured using a method known as Double Electron Electron Resonance (DEER) spectroscopy. The DEER method is based on the fact that the magnetic dipolar interaction between two spins depends inversely on the cube of the distance between them. The time domain signals were Fourier-transformed to give spectra similar to those shown in Figure 3A and 3C for the WT and M1 sequences. The spectra reflect a frequency distribution given by

$$\nu = \frac{\mu_0 g_1 g_2 \beta^2 \left(3\cos^2\theta - 1\right)}{4\pi h r^3}$$

where g_1 and g_2 are the isotropic *g*-factors of each electron, β is the Bohr magneton, μ_0 is vacuum permeability, *h* is Planck's constant, *r* is the interspin distance, and θ is the angle between the magnetic field and the interspin vector. The spectra exhibit the characteristic "Pake pattern" with turning points corresponding to $\theta = 0°$ and $\theta = 90°$ (*cf.* above equation) In addition, this pattern reflects the distribution of interspin distances *r*, which is a key feature of DEER spectroscopy that will enable us to map the H12 domain dynamics in response to different ligands.

We have labeled the ER-LBD in three key positions: (1) C447 in the body of the ER-LBD (a native cysteine), (2) C530 in the hinge region adjacent to H12, and (3) C543 on H12 itself. Distance measurements between these points allow us to triangulate the repositioning of H12 in response to the bound ligand. Our first studies have focused on optimizing experimental conditions in order to observe the intramolecular label distances of interest. This was necessary because the ER-LBD naturally forms a dimer, so that both

inter- and intra-molecular distances appear in the distance distribution. By measuring the fully labeled dimer as well as a 3-5 fold dilution of the fully labeled dimer with unlabeled protein (or protein-ligand) we were able to separately determine inter- and intra-molecular distances both in the presence and absence of estradiol (E2), as summarized in **Table 4.**. These results clearly reveal significant distance changes in both the position of H12 as well as the conformation of the dimer itself upon binding of the ligand.

Table 4 Distance measurements between label sites shown in

	530-530 Å	**530-447 Å**	**543-447 Å**
Apo	**24**	**42**	**30**
Holo (E2)	**20**	**36**	**37**

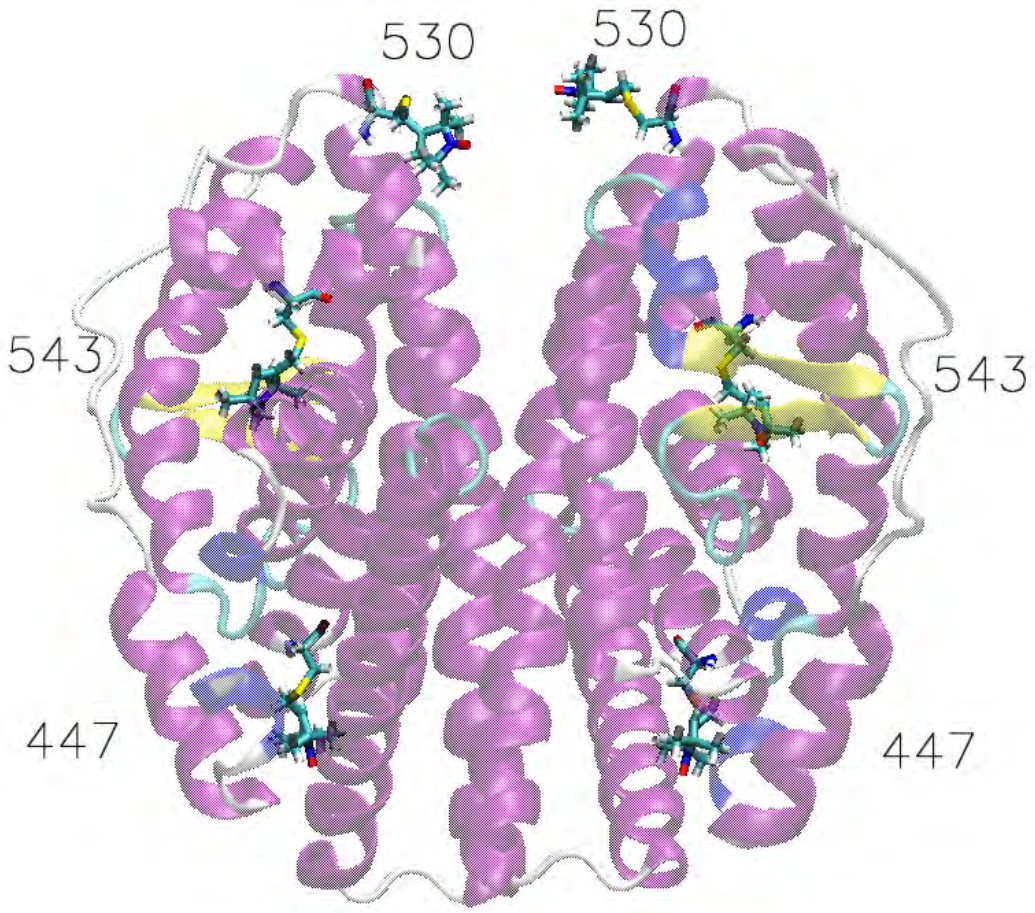

Figure 11 Summary of label positions on the ERα-LBD dimer for double-label distance measurements (only two labels are present at a time in a given experiment).

The initial DEER studies reported above were carried out in collaboration with the group of Prof. Peter G. Fajer at the National High Magnetic Field Laboratory and the Biology Department of Florida State University. Since the start of this work, our group has been awarded an instrumentation grant from the NSF (award DBI-732001) to construct a high-field DEER spectrometer at 230 GHz. We anticipate that the new instrument will come on line later this year and hope to apply it to carry out our distance measurements on ER in house.

3. Key Research Accomplishments

1. Completed synthesis of 17α- labeled estradiols needed for EPR studies.
2. Developed new assay for binding to ER based on EPR and fluorescence of 17α spin-labeled estradiols
3. Completed synthesis of first antagonist 11β-labeled estradiol
4. Optimized expression and purification of all ER-LBD mutants needed for spin-labeling studies, including doubly-labeled mutants
5. Quantified significant ligand-dependent dynamic changes in the hinge region between helix 12 and the body of the ER-LBD protein.
6. Completed initial distance measurements of doubly labeled ER-LBD by DEER spectroscopy demonstrating significant ligand-dependent changes in H12 region

4. Reportable Outcomes

Presentations at meetings

1. Stefano V. Gullà, Robert N. Hanson, and David E. Budil, *Site Directed Spin Labeling Study of Ligand Induced Estrogen Receptor Conformations*, 49th Rocky Mountain Conference on Analytical Chemistry, Breckenridge, CO, July 23-27, 2007
2. Stefano V Gullà, Robert N. Hanson, J. Adam Hendricks, Kalman Hideg,[2] and David E. Budil,[1] *New site-directed spin labeling tools for characterizing the dynamic response of the estrogen receptor to therapeutic agents*, 235th National Meeting of the American Chemical Society, New Orleans, LA, April 6-10, 2008
3. Stefano V Gulla[1], Kalman Hideg,[2] David E. Budil, *Characterization of spin labeled estradiol as a probe for Estrogen Receptor binding interactions*, 235th National Meeting of the American Chemical Society, New Orleans, LA, April 6-10, 2008
4. Samantha Rupert, Kelly Barhite, Stefano Gullà, David E. Budil *Spin label studies of interactions between the estrogen receptor and coactivator peptides*, Experimental Biology 2008 meeting, April 5-9, San Diego, CA, 2008
5. Stefano V Gullà, Robert N. Hanson, J. Adam Hendricks, Kalman Hideg,[2] and David E. Budil,[1] *New site-directed spin labeling tools for characterizing the dynamic response of the estrogen receptor to therapeutic agents*, 5th Era of Hope Meeting, June 26-30 Baltimore, MD, 2008

6. Stefano V Gullà, Jean Chamoun, Peter G. Fajer, Kalman Hideg, and David E. Budil, *New site-directed spin labeling tools for characterizing the dynamic response of the estrogen receptor to therapeutic agents*, 50th Rocky Mountain Conference on Analytical Chemistry, Breckenridge, CO, July 25-30, 2008

Manuscripts in preparation

1. Stefano V. Gulla, Kalman Hideg, Jean Chamoun, Peter G. Fajer, David E. Budil, *Characterization of novel estrogen-based nitroxide spin probe binding to the estrogen receptor α ligand binding domain*, in preparation for submission to *J. Mol. Endocrinol.* (2008)
2. Stefano Gullà, J. Adam Hendricks, Robert N. Hanson, and David E. Budil, *Spin-label study of ligand-dependent receptor dynamics in the ligand-binding region of estrogen receptor α*, in preparation for submission *Biochemistry* (2008)
3. Stefano Gullà, Jean Chamoun, Peter G. Fajer, and David E. Budil,*Solution structure of the dimer of the estrogen receptor alpha ligand binding domain by double electron electron resonance (DEER) spectroscopy*", in preparation for submission to *Biophys. J* (2008).

5. Conclusions

The project is proceeding on schedule according to the original Statement of Work. Tasks 1 (Synthesis of spin labeled estrogenic ligands) and 2 (Production of spin-labeled mutants) have essentially been completed. Task 3 (characterization by cw and double resonance EPR) is underway and has already provided significant information about ligand-dependent dynamics in the Helix 12 region of the Estrogen Receptor. The results clearly support our initial hypothesis that the physical response of the ER protein to different ligand types can be resolved and characterized in detail by EPR spin-labeling.

6. References

none